Position Paper:

Fertility intervention and toxicant technologies for the eradication of rodents on Lord Howe Island

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Abstract

Lord Howe Island's unique ecosystem is under threat from a number of introduced pest species including the Ship Rat (*Rattus rattus*) and the House Mouse (*Mus musculus*). This paper reviews novel and emerging strategies for rodent eradication, with a particular focus on the use of fertility interventions and sterilants as alternatives to traditional toxicants. While the majority of technologies associated with fertility interventions are still in developmental phases, this approach has a greater potential for species-specificity than toxicants, reducing the risk that off-target species such as humans, domestic and native animals will be affected. The strategies outlined in this manuscript include established technologies such as immunocontraceptives and gonadotoxicants, along with a host of developing technologies and delivery strategies to prevent the reproductive cycle and facilitate pest eradication.

While fertility interventions may indeed have the capacity to be a sole tool for eradication, they would need to be applied in sufficient volumes and at a high enough density to provide eradication as opposed to simply being a control measure. Despite substantial progress in the innovation of fertility-inhibiting agents, their deployment is hindered by difficulties in delivery, especially rodents where injectable agents are not a feasible option. Since the success of even the most effective fertility-inhibiting agents will depend on ability to deliver and disseminate them, novel developments and directions in this field are also discussed. Research into improved lethal strategies is ongoing; as the advantages and disadvantages pertaining to use of anticoagulant baits have been widely documented elsewhere, we report here only on novel formulations of non- anticoagulant toxins which have enhanced palatability, efficacy and delivery strategies compared to previous formulations, and strictly novel concepts. These range in their maturity from ideas briefly documented in patent applications to lab-tested compounds entering field trial and commercialisation phases. A series of applied evolutionary strategies have also been proposed and offer a new angle on mammal eradications, with proven efficacy in insect populations and theoretical modelling for vertebrate species indicating very likely eradication success. These approaches are presently limited by their novelty – in particular the absence of a robust regulatory framework and potential obstacles at the socio-political level.

Island eradications present an opportunity for incorporating new tools and paving the way for truly integrated and innovative conservation practices. Numerous advancements in this field, both disruptive and incremental, are currently in development but often endure a lengthy journey from lab to field with costs of commercialisation, regulatory constraints, socio-political controversy and biological challenges posing some of the obstacles. With the availability of sufficient resources, a concerted effort to combine known immunocontraceptive and gonadotoxic agents to facilitate contraceptive and sterilant delivery would likely yield a product that is logistically simple to deliver in conjunction with novel toxicants for the eradication of pest rodents within a 3-5 year time frame.

1. Fertility interventions for rodent eradication

Fertility intervention comes in many forms and has the potential to significantly reduce the environmental and off-target species impacts of lethal strategies. For invasive rodent species, fertility control strategies offer great potential because of the relatively short natural lifespan and high fecundity of these animals. Several established and emerging technologies for mammalian fertility control could contribute to an integrated eradication program; however with regard to rodents in particular, delivery of the sterilizing agent becomes a major challenge, given that most of the existing strategies rely on direct injection of a compound, which is not a feasible option in free-ranging rodent populations. The main requirement of a fertility control strategy, if it is to be relevant to pest rodents, is that it must have a means of administration that does not require capture of individual animals. Furthermore, an ideal scenario would involve self-dissemination of the fertility limiting strategy selected. Whilst delivery and dissemination of fertility control represent an entire field of innovation on its own, they are tightly intertwined with, and dependent on, the type of technology being delivered. In this section we discuss fertility intervention approaches that are already in use for some species but yet to be developed for free-ranging rodents, as well completely novel approaches currently under development and likely to emerge in coming years. The existing approaches include immunocontraceptives and gonadotoxicants (section 1.1), and are likely to persist into the future as central concepts in fertility control as there is much scope for improving their efficacy and adaptation for use in various species, including rats and mice. Several novel and potentially disruptive animal contraception technologies are also in the pipeline at research institutions and biotech firms around the globe (section 1.2). Although likely to still be several years away from beginning the commercialisation process, these are also discussed in some detail. Novel strategies include modifications in hormone production via single-shot administration of modified adenovirus vectors, reproductive toxins targeted directly to the gonads using receptor specificity, and genetic engineering for production of single sex offspring.

Whilst delivery and dissemination strategies are considered here as they pertain to each individual fertility control approach, the recent developments and innovations in this field are discussed in further detail in section 3 of the paper.

1.1 Contraceptive agents and sterilants used in mammals

Overview

For mammals in general, several non-surgical fertility control methods are presently available. These are grouped into steroid hormone-based contraception, immunocontraception and toxic agents (gonadotoxicants). Hormone contraceptives, although extensively used for human and domestic animal fertility control, are generally

considered unsuitable for free-ranging wild and feral animals because of safety concerns, environmental persistence of active metabolites, short duration of action, difficulty and cost of remote administration and behavioural effects. Immunocontraception, which involves vaccinating animals against their own reproductively functional antigens, has shown great promise in reducing fertility and continues to evolve as new targets are identified and vaccines are developed. Multiple immunocontraceptive formulations are already in use for wild and captive mammal populations around the world and are proving successful. Current limitations for use of immunocontraceptives in rodents revolve around difficulty in delivering and disseminating the vaccines, with a need to address the research gap at the stage of vaccine formulation beyond proof-of-concept and to develop alternatives to the injectable route for vaccine delivery. Fertility-limiting toxic agents derive from a range of different chemical groups and therefore vary in their safety and effectiveness profiles; these shall be discussed further in more detail. Of particular note is the combination of 4-vinylcyclohexene diepoxide and triptolide, which has recently gained regulatory approval in the USA specifically for use in rats.

Immunocontraceptives

Immunocontraception involves stimulating the animal's own immune system to raise an antibody response against a specific protein or hormone that is necessary for reproductive function. If the antibody response is of sufficient magnitude and duration, it will impair the normal action of the reproductively functional protein and inhibit successful reproduction in the target animal. In essence this is the vaccination of an animal against its own reproductive machinery.

The immunocontraceptives, or 'contraceptive vaccines', studied and developed to date can be grouped into three categories: those targeting gamete production, gamete function and the product of gamete fusion (gamete outcome). Initially, development of immunocontraceptives for wildlife focused largely on *gamete production;* specifically, gonadotrophin-releasing hormone (GnRH) vaccines, which work by preventing stimulation of the pituitary by GnRH, in turn reducing circulating gonadotrophic hormones (follicle-stimulating hormone and leuteinising hormone) and thus inhibiting ovarian and testicular function. GnRH vaccines are commercially available and have been deployed for temporary fertility control of domestic dogs (Donovan et al., 2012), cats (Robbins et al., 2004) and horses (Turkstra et al., 2005); however, their use in free-ranging animals has been limited due to practicality of administration (i.e. by intramuscular injection) and limited duration of efficacy. Incremental improvements in GnRH vaccine efficacy and delivery are likely to see this strategy persist in domestic and wild animal fertility management for many years to come. An important aspect of such improvements is increasing the immunogenicity of GnRH - a small, endogenous peptide that requires modification in order to elicit an effective immune response in the host. This has been achieved via various strategies including coupling the GnRH

peptide to larger, more immunogenic proteins or peptides (e.g. tetanus toxoid, keyhole limpet hemacyanin (KLH) or ovalbumin) (Junco et al., 2007) or by novel constructs that combine GnRH with immunogenic epitopes from infectious microbes (Talwar et al., 2004). The ability to deliver the GnRH vaccine via an oral (baiting) route, rather than by direct injection, will be paramount to its usefulness in managing free ranging populations, in particular rats and mice. This has been the subject of several years of recent research at the CSIRO and other research groups. Thus far, mucosal GnRH immunisation has been achieved only via nasal aerosol delivery (Gebril et al., 2014).

The most advanced examples of *gamete-function* targeting vaccines are those targeting the zona pellucida (ZP), which is the outer glycoprotein layer located of the mammalian oocyte (egg) and functions as the primary interface for sperm-egg interaction. Antibodies against ZP surface peptides cause infertility by preventing penetration of the ZP by spermatozoa and thus inhibiting fertilisation of the oocyte within. Zona-targeting vaccines carry some advantages over GnRH vaccines, such as small delivery volume and the retention of normal reproductive behaviours. Currently, injectable zona pellucida vaccines are capable of reducing fertility in mammals for up to 3 years following a single administration (Gray et al., 2010), but the inability to deliver this vaccine by means other than injection is clearly a prohibitive limitation for use in free-ranging rodents. We also note here that, although the vaccine's effects are temporary in most cases, there have been observations of disrupted ovarian function and depletion of the primordial follicle pool following ZP vaccination treatments (Joonè et al., 2017; Paterson et al., 2002). Whilst this has been considered a major disadvantage in the context of developing a temporary and reversible human contraceptive, the phenomenon could be advantageous for animal fertility interventions and requires further investigation as it provides scope for a long-term or even permanent contraceptive for free ranging invasive species.

Fertility interventions targeting *gamete outcome* are a relatively recent development and somewhat beyond the conventional definition of 'contraception' – such methods would inhibit continuation of pregnancy, rather than prevent conception. The most prominent example in this category has been the human chorionic gonadotrophin (hCG) vaccine. This vaccine targets the active subunit of hCG (the human pregnancy hormone) and has undergone a series of successful trials. Shown to be efficacious and safe, it has now been deemed a promising new birth control method for humans (Talwar, 2013). Unfortunately, the method is limited to use in humans (and other primates) since the hormone is only present in these species, thus its application evidently cannot extend to wildlife.

Aside from GnRH and ZP, many potential targets for immunocontraceptive vaccines are under active investigation and novel targets are steadily emerging as new information is gathered about the roles of specific biomolecules in gamete maturation, fertilisation and early embryonic development (Frank et al., 2005; McLaughlin and Aitken, 2011; Menkhorst et al., 2008; Swegen and Aitken, 2014). Immune-based

contraceptive strategies will likely remain a keystone of future research and wildlife management, however must occur in synergy with innovations pertaining to improved formulations for delivery (in particular oral delivery), dissemination and prolonged duration of efficacy. This is of particular concern for rodent applications, where delivery by injection is not an option, and the strategy, no matter how effective in other species, cannot be deployed unless an effective means of delivery is developed alongside it.

While safe and effective zona pellucida and GnRH vaccines are available for use in wildlife, these are injectable formulations unsuitable for use in free ranging rodents. These immunocontraceptives are only as good as their delivery and dissemination strategies, and, at this stage, with viral vector delivery research halted and oral/mucosal delivery still at least several years away, there are no immediate prospects for a commercially available rodent immunocontraceptive. With concerted effort into improving mucosal delivery and species-specific design of 'immunisation stations', rodent immunocontraception could be feasible within 5-10 years. Unfortunately no such research programs appear to be underway presently.

Gonadotoxicants

Toxic agents targeting the gonads, or gonadotoxicants, referto chemicals capable of inducing infertility via premature failure of spermatogenesis, ovarian pathology or primordial follicle reserve depletion. In general, the chemicals rely on the irreversible destruction of targeted tissues, resulting in permanent infertility. Whilst several such compounds have been known for many years, the majority are not specific to the reproductive system and cause adverse systemic effects unless coupled with a targeting strategy; thus developing a safe, effective and palatable formulation for free-ranging animals has proved a challenge (Amory et al., 2014). Nonetheless, these obstacles are gradually being conquered, at least in the case of rodent fertility control: the first anti-fertility formulation for large scale use in rat control, ContraPest®, was registered and approved for use in the USA in 2016 (senestech.com/rodent-control-our-product/). The formulation consists of an orally palatable bait containing the chemical 4-vinylcyclohexene diepoxide (VCD), which causes depletion of non-regenerating primordial ovarian follicles (Hoyer et al., 2001), together with triptolide, a Chinese herb that induces apoptosis in growing follicles (Dyer et al., 2013). Male fertility is also impaired by VCD via apoptosis of developing germ cells and testicular degeneration (Adedara et al., 2016; Hooser et al., 1995). ContraPest® is claimed to be species-specific and safe for human handling, with no risk of bioaccumulation due to the short half-life of the active compounds facilitated by rapid metabolic breakdown and environmental breakdown into inactive products. However, this species specificity is presumably achieved via the low concentrations of active compounds, limiting possible effects on larger domestic mammals, and the design of the proprietary bait stations, which only allow access to

animals matching the size profile of the rat. Rats need to repeatedly access baits and eat 8-10% of their body weight for 5-10 days to attain reduction in litter sizes within 4 weeks, progressively attaining sterility at 8-12 weeks of consumption. Marketing of the product has thus far been targeted toward controlling, rather than eradicating, populations of urban rats and it is unclear whether similar-sized mammalian wildlife species would be affected if the baits were deployed in rural or wilderness areas. ContraPest® has been approved by the US EPA for use in rats. It entered the registration process with the APVMA in 2008, though its current status is pending, presumably due to recent modification and improvement to the formulation.

In addition to gonadotoxicants that induce long-term damage to the germ line, a number of compounds have been investigated over the years for their use as reversible contraceptives interfering with sperm function, at a post-testicular level. Interestingly, several showed promise in rodent trials (in particular rats) but were less effective in humans, likely due to differences in systemic and sperm metabolism across species. In many cases when contraceptive success did not translate beyond the rodent model, further investigation was not pursued. One such example is ornidazole, a commonly used antibiotic and antiprotozoal drug. The compound was the subject of investigation as a putative male contraceptive in the 1980s and trials in rats demonstrated a capacity to induce complete, temporary infertility via inhibition of sperm glycolysis and subsequent reduction of sperm motility following oral administration (Cooper and Yeung, 1999; Oberlander et al., 1994). While the drug is considered safe for human consumption in short-term treatment protocols as an antibiotic/antiprotozoal, the doses required to maintain a contraceptive effect in men were considered too high to be practicable in a chronic exposure setting. Given the apparent sensitivity of the rat to this compound and substantial progress in strategies for controlled release and targeted delivery of drugs over the last 3-4 decades, revisiting ornidazole and other glycolysis-inhibiting compounds may be worthwhile in the context of rodent fertility control.

A more recent approach to gonadotoxicant development involves increasing effectiveness while minimizing systemic side effects by combining toxicants with targeting strategies that facilitate the delivery of the compounds to a specific tissue or cell type. These approaches are discussed in section 1.2.

1.2 Novel fertility intervention strategies under development

Overview

A number of technologies are under development that seek to sterilise mammals, in particular dogs and cats, with a single injection. These vary in their stages of research but many are showing promising results thus far. Projects include developing strategies to silence genes essential for reproductive function using a range of vectors,

innovations in immunocontraception, and targeted delivery of cytotoxins to various levels of the hypothalamic-pituitary-gonadal axis. The majority of these projects utilise mice and/or rats as their model species and it is highly likely that, once proof-of-concept stage is complete, they can be adapted to various species including the rodents by means of adjusting dosage and formulation of the newly developed compounds for optimal delivery to given species.

In addition, a series of patent applications have been recently registered pertaining to new compounds for fertility control of rodents; not all of these were approved but these patent applications provide insight into some of the lesser known strategies under development. These have varying mechanisms of action but most are based on plant derived chemicals and draw on knowledge of herbal remedies used in traditional Chinese medicine.

A novel approach to immunocontraceptives

Although immunocontraceptives which target various antigens have proven to be safe and effective in reducing fertility, one of the main setbacks of using immune-based strategies in free-ranging animals has been the limited duration of efficacy. This stems from the challenges of eliciting a strong immune response against the animal's own ('self') antigens, i.e. low immunogenicity. Long-term, targeted autoimmunity appears to be particularly difficult to induce. Despite this, the prevalence of human autoimmune diseases such as type 1 diabetes, lupus and coeliac disease are increasing. These diseases are characterised by a persistent immune response against the host's own antigens, resulting in destruction of functional proteins and the malfunction of key physiological processes. While the aetiologies of these conditions remain complex and uncertain, significant progress has occurred in research seeking to understand what can cause such an aggressive and sustained autoimmune response, i.e. what processes underlie increased immunogenicity of 'self' antigens. One hypothesis is that the antigenic proteins become more immunogenic when they are damaged, or changed in some way and are then presented to the immune system. The most effective types of modification required to elicit this response are still under investigation, though a variety of lipo- and glyco-oxidative modifications have been shown to generate 'neo-antigens' which are associated with increased immunogenicity of the blood protein serum albumin (Jyoti et al., 2016; Khan et al., 2016) and DNA (Alzolibani et al., 2014; Wang et al., 2015). Given the susceptibility of spermatozoa to oxidative damage (Aitken et al., 2012a; Aitken et al., 2012b), Aitken et al. have proposed that some of these biochemical modifications could be exploited in order to increase the immunogenicity of sperm proteins and would facilitate an improved and long-lasting contraceptive response. This hypothesis is presently the subject of a proof-of-concept study being carried out in mice; preliminary results have indicated that at least one protein modification results in increased immunogenicity of sperm proteins and can generate infertility. This approach to animal contraception offers both the

established safety profile of immunocontraception and the transformative aspect of incorporating new knowledge from fields outside of reproductive biology. It is currently expected that further development of this approach will make a new immunocontraceptive available for commercialisation within the next 5 years.

Using gene therapy expressing Müllerian Inhibiting Substance to induce sterility in mice by blocking follicle recruitment and testosterone production

Exciting data are emerging from the lab of Dr Donahoe and Dr Pepin in the USA, who report having successfully induced sterility in female mice with a single injection (Pepin et al., 2016). Their strategy involves modifying the production of Anti-Müllerian Hormone (AMH; or Müllerian Inhibiting Substance - MIS) such that follicular recruitment is completely halted (Pepin et al., 2015). AMH is produced in the granulosa cells of the ovary and is best known as a predictor of mammalian females' ovarian reserve. At higher than normal levels, this hormone inhibits the recruitment of oocytes into the ovulatory cascade, thereby preventing ovulation and the ability to conceive offspring. The novel approach has been to dissect the genomic sequence of the mouse to better understand the production of AMH, and then generate an adeno-associated viral vector that will stimulate production of MIS (a recombinant form of AMH) when injected into subject animals - thus increasing MIS production to the point that ovulation, and therefore fertility, is completely suppressed. The technology is expected to be highly species-specific as it relies on each species' individual genome to be successful, and should provide life-long sterility, or at worst long-term contraceptive effects. Whilst clearly a promising development, a limitation of the technology for use in rodents - in its current form - is the need for injection into individual animals. Furthermore, the strategy has been worked up in the mouse but would need additional input to achieve the same outcomes in rats due to its species-specific nature. It is unclear whether the lab will be pursuing commercialisation of the technology for rodent species or only domestic/stray animals such as dogs and cats.

Permanent germ cell ablation by peptide-targeted delivery of reproductive toxicants

Research at the University of Newcastle's Priority Research Centre (PRC) in Reproductive Science has focused on development of compounds that will selectively target reproductive tissues and induce permanent sterility by localised cytotoxicity. Several years of research were dedicated to identifying chemical structures that would navigate a cytotoxic compound toward cell types unique to the reproductive systems of male and female animals, i.e. primordial oocytes, spermatogonia and Sertoli cells. These cells are not only specific to the reproductive system but are vital to reproductive functioning and non-renewable, so their selective ablation is expected to result in complete and irreversible infertility.

Using phage peptide display technology, we have identified a panel of peptides that are able to selectively bind to primordial oocytes, spermatogonia and Sertoli cells. Coupling these peptides with redox cycling xenobiotics should ensure selective apoptosis of the target cells and lead to sterilisation. Selective cell death has been demonstrated *in vivo* (in mice) and the next stage of research is underway; a selection of cytotoxic compounds are now being coupled to the identified targeting peptides and trialled in mice to determine an optimally safe, potent and appropriately dosed toxicant-peptide combination that will result in applied infertility. In addition, peptides have been designed that bind to the follicle stimulating hormone receptor (FSHR), providing yet another means of specifically targeting only the reproductive system. These have now been demonstrated to bind to the FSHR *in vivo* and thus target Sertoli cells and granulosa cells. Trials are now taking place to test effectiveness of various cytotoxin-peptide combinations.

The estimated target date for commercialisation of these compounds is late 2018, however this pertains to an injectable, single-shot sterilant for domestic mammals and therefore other formulations would need to be worked up in order for this technology to be a feasible option for rodent control (e.g. oral baits); there are currently no immediate funding provisions for this commercialisation route.

Modification of bacterial toxins to target the reproductive system via activation by specific cell surface proteases

Another approach to non-surgical sterilisation under development in the University of Newcastle's PRC in Reproductive Science exploits the advances resulting from the "arms race" between pathogenic bacteria and their hosts. Many species of bacteria have evolved mechanisms whereby the pathogen is able to enter its host undetected but can then cause significant damage having bypassed the earlier phases of the immune response. Part of this strategy involves the release of inactive forms of a toxin by the bacteria; the inactive form is subsequently cleaved by a specific protease found on the surface of the host's cells. This cleavage allows the toxin to enter the host cell, become activated and induce cell damage and/or cell death. The activation domains of such toxins can be modified to be activated only by proteases found only on the surface of cells within the reproductive system. For example, the serine protease testisin (PRSS21) is almost exclusively expressed by testicular germ cells and tumours of ovarian origin (Hooper et al., 1999; Shigemasa et al., 2000). In the mouse this protein is expressed in the membranes of developing sperm cells within the testis (Scarman et al., 2001) and in epididymal spermatozoa, and is anchored to the outside of the cell membrane (Honda et al., 2002; Nixon et al., 2009). Importantly, testisin is enzymatically active and can function as a protease at the cell surface or extracellular space (Tang et al., 2005) – the same trait that is often exploited by toxin-producing bacteria (Gordon and Leppla, 1994). This feature has been harnessed by cancer researchers who are seeking to utilise proteolytic mechanisms to design

cancer-destroying toxins which have their substrate domains modified so that they may be activated specifically by cancer cell-expressed surface proteases. Testisin has been the focus of such a strategy, being a tumour- and testis- specific protease. Antalis et al. have successfully modified a recombinant anthrax toxin in such a way that it is activated when it comes into contact with testisin on the tumour surface, facilitating entry into and destruction of the cell (Martin et al., 2015). Research within University of Newcastle's PRC in Reproductive Science is underway to determine whether the proteolytic activity of testisin and other reproductive system-specific surface proteins can be used to direct modified bacterial toxins to the gonads and inhibit reproductive function.

Lentiviral-mediated miRNA suppression of the androgen receptor in Sertoli cells

The lab of Dr Smith at the University of Edinburgh has taken the innovative approach of designing microRNA sequences that, when delivered in a targeted manner to Sertoli cells in the testis, would effectively suppress androgen receptor production, thus removing the Sertoli cells' ability to respond to testosterone—which is necessary to stimulate production of spermatozoa.

These miRNA constructs have been designed and have demonstrated effectiveness in vitro; furthermore, lentiviral particles have been engineered that are able to carry the miRNA into cells by binding to FSH receptors to gain access specifically to the Sertoli cells (Roesl et al., 2016). The technology has undergone preliminary trials in male mice and showed suppression of fertility for at least 5 months, demonstrating great promise as a single-shot non-surgical sterilant. The researchers have recently initiated a new project which will seek to further refine the delivery of the sterilant and are set to begin a clinical trial in cats and dogs, due to be completed in 2020.

Sophoricoside

Sophoricoside is an isoflavone glycoside isolated from the fruits of Sophora japonica. One published study reports that 600 mg/kg per day administered to mice for several days induced changes in uterine morphology and reduced uterine receptivity, preventing embryo implantation. The authors observed up-regulation of uterine progesterone receptor and down-regulation of estrogen receptor α along with several implantation factors (Zhou et al., 2014). A patent for the use of this compound as a rodent anti-fertility agent claimed that "compared with a traditional ecological deratization method and chemical poison bait method, the breeding resisting method has long effective time, obvious control effect, small toxic and side effects on the environment and the like, and can more effectively inhibit the super compensatory

effect of rat population" (Zhang et al., 2014a), however data to support these claims are not published. The patent documentation reports that when sophoricoside is administered orally for 5 consecutive days the infertility rate of female rats can reach 70%. It is not known whether work to further develop this product as a commercial product is continuing.

Arecoline hydrobromide

Arecoline is a nicotinic acid-based alkaloid found in the areca nut, the fruit of the areca palm (Areca catechu). It is the active ingredient responsible for the tobacco-like effects of betel nut chewing, common in parts of India and China. Authored by the same group as the sophoricoside anti-fertility agent, a patent application for the use of arecoline has been recorded. The authors make a similar claim about the potential of this compound as a rodent control technology: "The antifertility technology is a novel mouse control technology developed in recent years, and compared with a traditional mouse control method, the technology has the advantages of long action time, obvious control effect, small environmental toxic and side effects and the like; therefore, the antifertility technology has great utilisation potentiality in the aspect of controlling the population of mice" (Zhang et al., 2014b). However, rigorous evidence to support this claim is lacking, and it is unclear how such a treatment would be delivered in urban or field conditions.

Numerous studies report the adverse health effects of consuming arecoline during pregnancy but no studies could be located reporting its potential as an applied strategy for rodent fertility control. For example, betel nut chewing during pregnancy has been associated with low birth weight and is considered a risk factor for poor pregnancy outcome (Senn et al., 2009). Arecoline has been shown to affect embryo implantation and development, decreasing the number of implanted embryos in early pregnant mice (Liu et al., 2011); the mechanisms for its action are undetermined.

Neem oil extract

Extract from the neem flower has been suggested as a potential contraceptive agent; oral administration of the extract has been shown to disrupt the oestrous cycle and ovulation in rats (Gbotolorun et al., 2008). This appears to be driven by a reduction in circulating levels of luteinising hormone, but the exact mechanisms responsible are unknown (Owolabi et al., 2008). It has been postulated that aqueous neem leaf extract induces reactive oxygen species-mediated granulosa cell and follicular oocyte apoptosis, and deterioration in oocyte quality is the main driver for fertility suppression (Chaube et al., 2014). In male mice, neem extract caused reversible changes to spermatozoa including adverse effects on motility, morphology, and concentration of

spermatozoa (Mishra and Singh, 2005). Neem seed extract is also reported to be effective in terminating early pregnancies after oral administration in rats and was even suggested as a potential new human contraceptive (Mukherjee et al., 1999). It is not known if further research is underway to develop a commercial anti-fertility agent palatable to rodents.

2. Genetic engineering for gender bias and sterility

Overview

Genetic engineering of invasive species populations, including both plants and animals, is an emerging strategy for eradication (Campbell et al., 2015; Sutherland et al., 2014). Modelling studies examining the interactions between population dynamics and altered population genetics have been carried out to assess the potential of several recombinant approaches, indicating that it would be feasible to introduce into pest species populations a genetic construct that distorts operational sex ratios (Bax and Thresher, 2009). Such a construct could sterilise one gender while being inherited through the other; this approach is expected to be highly effective as a means of pest control and to be resilient under a variety of ecological and behavioural conditions. Multiple genetic strategies have been proposed and modelled for mammalian pest control and the most prominent are briefly outlined in the proceeding sections.

Trojan female technique

This strategy draws on the evolutionary hypothesis that the mitochondrial genome is prone to the accumulation of male, but not female, harming mutations. More specifically, it is proposed that mitochondria, inherited through the maternal lineage only, can determine the quality and function of spermatozoa in the male. Mutations in mitochondrial DNA (mtDNA) can impair sperm motility and render the host male infertile (Dowling et al., 2015). Separating 'fitness' (in this case, fertility) from inheritance is the key factor in the success of genetic strategies as this allows the modified population to dodge natural evolutionary processes; maternal inheritance means that mtDNA sequences are only directly affected by natural selection when carried by females, and will not be eliminated from the population if they do not reduce fitness in the female phenotype (Gemmell et al., 2004). These mechanisms are no longer the realm of pure theory and have been validated experimentally in *Drosophila melanogaster* (Innocenti et al., 2011). This somewhat ironic twist of evolutionary fate can be exploited by genetically engineering female animals carrying male-sterility mtDNA mutations; the male offspring of these females would be sterile while the female offspring would inherit the modified mtDNA construct and pass it on to the next generation, eventually resulting in a female-only population unable to continue

reproducing.

While the Trojan female and other genetic strategies are most commonly discussed in the context of insect population control (in particular, disease-transmitting mosquitoes), mtDNA-associated infertility has been reported in mammals, especially those species where significant naturally occurring infertile populations exist (Ambulkar et al., 2016; Hayashida and Kohno, 2009; Hosseinzadeh Colagar and Karimi, 2014; Song and Lewis, 2008; Spiropoulos et al., 2002). Furthermore, the modification of mtDNA has effectively generated male infertility in the mouse (Nakada et al., 2006), lending a plausible foundation to further development of the Trojan female technique in rodents and other mammals. Other candidate Trojan female mutations will need to be identified and experimentally validated before progress can be made on the practical development of this strategy in mammals, highlighting the need to better understand fundamental mechanisms of mammalian reproductive biology and the capacity of mtDNA to determine male fertility. In theory, this eradication strategy would be applicable to any species in which such mutations can be identified, including rats and mice. While modelling and laboratory experimentation have indicated the Trojan female strategy to be promising, certain conditions (both genetic and ecological) will need to be met in practice in order to confirm its potential to successfully eradicate pest species (Gemmell et al., 2013). Evidently, extensive experimental work is yet required to make this strategy a realistic option for mammalian pest control.

t-Sry strategy

Another genetic engineering strategy, proposed and modelled specifically for eradication of invasive mouse populations, describes a modification for gender bias towards a male-only population (Backus and Gross, 2016). In a sense the t-Sry strategy mirrors the Trojan female strategy: female sterility is inherited through the paternal lineage. The male-only Y chromosome contains a gene called Sry (sex-determining region of the Y chromosome), which is responsible for development of a male phenotype, i.e. development of testes. Whilst normally only carried on the Y chromosome and therefore only present in males, the Sry gene can be inserted into an autosome and therefore transmitted to females. This results in a male phenotype occurring in XX (female) individuals; these individuals are sterile since they lack the additional physiology necessary to produce a fully functional male reproductive system. On the other hand, the XY (genuine male) offspring will be fertile but will inherit and continue to pass on the modified Sry construct. Successful transmission of the Sry construct is ensured by coupling the Sry construct to the t-haplotype, which underpins the male transmission ratio distortion mechanism in the mouse, and ensures that males transmit the t-Sry construct to 90% of their offspring (Schimenti, 2000).

Similarly to the Trojan female technique, this strategy relies on the assumption that

the true males carrying the t-Sry modification will be equal in phenotypic fitness to wild-type males, i.e. that natural selection will not eliminate these males from the population, allowing the modification to persist in the population and ultimately leading to a male-only population unable to continue reproducing. Population modelling of the t-Sry strategy in mice has indicated that it would lead to a successful eradication; however the strategy requires that a relatively large number of modified individuals be released in order to inundate the existing population of invaders and successfully spread the introduced gene (Backus and Gross, 2016). Furthermore, it is likely that multiple releases would be required in the realistic scenario that modified mice would be slightly less 'fit' than wild type animals, since natural attrition of the modified population would occur over several generations. A potential solution to this is to simultaneously improve fitness of the modified mice, but this approach raises concerns over the controllability of the eradication process and the risks associated with the potential escape of an animal into a non-target population, which could lead to unintended extinction of the population. A less risky approach to reducing the ecological impact of releasing large numbers of additional mice would be to combine the strategy with a conventional rodenticide baiting campaign or timing the release in accordance with natural variations in mouse populations. Such an approach to eradication is likely to be synergistic, as a baiting campaign would allow for a significant reduction in numbers but would avoid the environmental and labour costs associated with ensuring complete eradication relying on rodenticides alone, while the genetic strategy would provide a self-perpetuating means of removing remaining animals. Engineering gene drives is yet another alternative to inundation release (discussed below). Clearly, the t-Sry strategy will require additional modelling of integrated approaches specific to each eradication scenario, thorough planning and ethical considerations. A thorough knowledge of the mouse populations, including seasonality and continuity/interbreeding data, would be absolutely vital to the success of any modified mouse release program.

Gene drives

The Trojan female technique and the t-Sry strategy are just two amongst many proposed and documented approaches to genetic engineering of pest (or pathogenic) species populations (Gould, 2008). An older approach, the sterile male technique, has been used for many decades in the control of malaria-transmitting mosquito populations (Benelli et al., 2016), and was successfully utilised to eradicate the screwworm *Cochliomyia hominivorax* in North America. The sterile male technique involves the sterilisation and release of a large number of male insects, which then mate with females but fail to produce offspring, and the strategy relies on the release of a relatively large number of males (greater than the target population) in order to achieve significant reduction in numbers. Similarly, an inundative release would also be required if a population of genetically modified rats or mice is to have any chance of

integrating the modified gene into the existing population; this is a factor of mating interactions and the natural rate of dispersal of the gene via Mendelian inheritance. However, gene drive technologies are likely to soon be available that will have the capacity to dramatically increase the rate of spread of such a gene through the population.

A recent development in gene drive technologies is based on the CRISPR-Cas9 system; this is a bacteria-derived endonuclease system that can be used to cut and insert DNA sequences in a target organism using an RNA guide. It has recently emerged as a game-changing technology within the field of genetic engineering and has already been utilised in a wide range of experimental and commercial applications owing to its simplicity and precision. When engineered to work as part of a gene drive, CRISPR-Cas9 has the ability to enhance dispersal of the target gene by inserting the gene in into the DNA in place of its alternative allele, making them homozygotic for the desired trait, and thus ensuring the target gene is inherited by the next generation. The principle has been demonstrated in practice, in the malaria vector mosquito *Anopheles stephensi*, where a CRISPR-Cas9 gene drive was used to disperse an anti-malaria effector gene; the anti-malaria effector constructs and marker genes were successfully introgressed into ~99.5% of the population when transgenic animals were crossed with wild-type animals, demonstrating the remarkable efficiency of this system (Gantz et al., 2015).

Exploiting gene drive techniques in combination with gender bias strategies for rodents would be extremely useful not only in achieving maximum dispersal of the target gene in the shortest possible time, but also in reducing the need for inundative release of additional rodents into the ecosystem, minimising the ecological impacts associated with the temporary increase in rodent populations, and significantly reducing costs. Although CRISPR-Cas9 and other genetic biocontrol technologies are generating much excitement in the conservation arena, researchers agree that significantly more research is needed to assess and address the risks associated with implementation of these novel strategies (Esvelt and Smidler, 2014; Webber et al., 2015). As mentioned, the transfer of a single animal to a non-target population of the same species can have disastrous consequences and even a minute level of risk calls for thorough development of biological and regulatory safeguards.

3. Delivery of contraceptive or sterilant interventions

Route of administration

The route of administration of an immunocontraceptive or gonadotoxic peptide is a critical factor in any intervention program. Proteins and peptides have poor

permeability characteristics. This if further exacerbated by the barriers presented by the gastrointestinal tract's (GIT) luminal brush border, cellular metabolism and degradation of the proteins. Should the proteins and peptides manage to be absorbed from the GIT into the blood stream in an intact form, they will then face the challenge of hepatic degradation by the liver and subsequent excretion from the body. These factors result in the poor bioavailability of proteins and peptides from oral routes, and as such, these agents are most effectively administered by parenteral routes such as intramuscular or subcutaneous injection (Muheem et al., 2016), or transcutaneous application (Engelke et al., 2015).

Oral delivery (baits)

Proteins and peptides are inherently unstable. The efficacy of these molecules as vaccines or toxins is largely due to their complex structures which are highly susceptible to alteration under a variety of chemical and physical stressors, resulting in a loss of biological activity. In addition, many proteins and peptides have very short biological half-lives as a result of the body's rapid clearance mechanisms including removal via the liver and metabolism by other body tissues through the action of enzymes and protein-modifying chemicals (Muheem et al., 2016). Enzymatic degradation of vaccines and peptides by peptidases and proteases of the GIT brush border and lumen respectively present a significant challenge to the oral delivery of these agents (Muheem et al., 2016).

Despite the aforementioned limitations to oral delivery of contraceptive vaccines and peptides, research into this route of administration continues to be actively pursued due to obvious advantages such as ease of administration, reduced costs associated with the treatment of large numbers of animals, and the lack of a need for sterile manufacturing facilities (Muheem et al., 2016). In order to meet the challenge of supplying an orally deliverable contraceptive intervention, an approach must be taken in which the vaccine or peptide is not only protected from enzymatic degradation in the GIT, but in which its absorption is enhanced without altering its biological activity (Gupta et al., 2013). Strategies are being actively developed to increase peptide bioavailability, overcome enzymatic degradation by the GIT, enhance peptide permeability and develop safe, efficacious and highly-potent proteinaceous drugs and vaccines (Hamman et al., 2005; Shah et al., 2002).

The initial sections of the GIT (being the stomach and the duodenum of the small intestine) are the primary sites of protein digestion, and the absorption of intact proteins following digestion is typically less than 1% (Iyer et al., 2010). As such, a proteinaceous contraceptive vaccine or peptide would need to be protected from degradation until it passes through these regions. One approach is to use a thick enteric coating over the formulation so that the agent is not released until it reaches the ileum and the colon (Rubinstein, 1995). An alternative approach is the use of

enzyme inhibitors such as aprotinin and soybean trypsin inhibitors, camostat mesilate and chromostatin (Muheem et al., 2016). A number of carrier systems such as emulsions, nanoparticles, liposomes, microparticles and microspheres can protect protein formulations against the harsh GIT environment and can significantly enhance absorption up to 5-fold (Carvalho et al., 2010; Chan et al., 2010; Gupta et al., 2013; Park et al., 2011; Sakuma et al., 2001), and a nanoparticle-delivery system for an oral sterilant for the control of brushtail possums in New Zealand is being developed with promising in vivo results to date (Kafka et al., 2011). Research at the National Wildlife Research Center (NWRC), USA, employed micro-encapsulation, protein stabilisation and selective pH release in order to develop an orally deliverable GnRH vaccine. Although protein stabilisation and selective pH release phases were successful, micro-encapsulation proved difficult. An adequate antibody response could not be achieved and it was concluded that future studies should focus on vaccine uptake via the transmucosal or nasopharyngeal route.

Due to poor lipophilicity, proteins and peptides cannot be passively absorbed across the membranes of cells lining the GIT, and instead will most commonly utilise a paracellular pathway (between cells), which is restricted to small molecules less than 100-200 Da in size (Camenisch et al., 1998). Disruption of the lipid membrane of the cells lining the GIT has been shown to significantly enhance peptide permeability, with the most efficacious approach being to attach the peptide or protein to molecules which open the tight junctions between adjacent cells (Brayden and Mrsny, 2011). In addition to tight junction penetration enhancers, surfactants (compounds with a detergent-like activity) and chelating agents are also able to enhance protein and peptide uptake by disrupting the lipid membrane and making the epithelial cells more permeable (Aungst, 2000; LeCluyse and Sutton, 1997; Park et al., 2011). There is an alternative to disrupting the structure of the GIT epithelial cells to facilitate permeation of the lipophobic agents across the GIT wall. By administering the vaccines or peptides with lipophilic carriers, their lipid solubility can be altered which allows their transport across intact GIT epithelial cell membranes (Leone-Bay et al., 2001; Sood and Panchagnula, 2001).

The GIT is lined with a viscous mucus which interferes with the diffusion of peptide drugs and vaccines across the GIT wall (Camenisch et al., 1998). As this mucus inhibits the uptake of proteinaceous agents, one approach to overcome this boundary is the use of the enzyme hyaluronidase to break up the mucus and allow the vaccine or peptide to directly contact the GIT wall with minimal interference, significantly increasing uptake of the contraceptive agent. An advantage of this approach is that it does not appear to have any detrimental effect on the integrity of the intestinal epithelial cells (Aoki et al., 2005). An alternative approach is to bind the vaccine or peptide to a mucoadhesive polymer which adheres to the mucus, increasing the concentration gradient at the epithelial cell surface and increasing availability for uptake (Muheem et al., 2016). In addition, the mucoadhesive nature of these polymers prevents the rapid clearance of the peptide or vaccine through the GIT, increasing

residency time and bioavailability (Gupta et al., 2013).

Transcutaneous delivery

Another parenteral route of immunocontraceptive vaccination is via the application of an ointment or liquid directly to the skin (transcutaneous). The possibility of transcutaneous immunisation against a number of protein, peptide and DNA antigens has been reported (Seid Jr et al., 2012), and lower doses of vaccine may be used compared to intramuscular injection (Engelke et al., 2015). The mechanism of action of transcutaneous vaccination is that upon presentation of an antigen in the vaccine, epidermal antigen presenting cells in the skin mature and migrate toward the draining lymph nodes, leading to a potent mucosal and systemic immunoglobulin response, coupled with robust cellular immunity (Ita, 2016). These antigen presenting cells and migratory T-lymphocytes, collectively called skin-associated lymphoid tissue make up the skin-immune system, and the extraordinary immunocompetence of the skin, coupled with its ease of access, makes this organ attractive for vaccination (Marshall et al., 2016). One complication associated with transcutaneous vaccination is that in order to increase vaccine efficacy, the skin barrier must be disrupted in some way, generally mechanically or chemically, so as to improve absorption of the vaccine and generate local inflammation which will also attract effector immune cells to the site of application (Engelke et al., 2015). While this somewhat limits the scope for the transdermal application of immunocontraceptive and sterilant peptide treatments in a free ranging rodent species, there is reasonable evidence to suggest that passive targeting strategies (involving simple application to the skin without any need for disruptive pre-treatments) may be effective if the contraceptive peptide or protein constructs are smaller than 500 Da. This is quite encouraging, given that one of the most effective immunocontraceptives, the ZP3 vaccine, is less than 100 Da, and therefore this may be a viable strategy to investigate for its delivery on a mass scale.

Mucosal delivery (aerosol)

The mucosal delivery of an immunocontraceptive via inhalation of an aerosol (intranasal) presents several advantages over intramuscular injection. The obvious advantage is that automatic aerosol dispensing devices can administer the vaccine without the need to individually handle each animal. Additionally, mucosal vaccine delivery produces a greater immune response with reduced antigen exposure. The reason for this is that the antigen presenting cell content of the muscle is poor and the co-stimulatory molecules absent, meaning that the overall immune response is reduced (Romero and Morilla, 2011).

Intranasal administration of aerosol vaccines is not without risk. This route of

administration enables uninterrupted nose-to-brain delivery as the antigen and adjuvant directly contact the primary olfactory neurons which communicate directly with the brain (Durrer et al., 2003). For this reason, intranasal vaccination poses an inherent risk of neurotoxicity (Mutsch et al., 2004) and as such, the stimulation of an immune response in the absence of a neurotoxicological effect presents something of a challenge for progress in the field of intranasal vaccine delivery research (Romero and Morilla, 2011).

An intranasal GnRH immunocontraceptive vaccine has been developed to reduce testosterone levels in prostate cancer patients. This has been achieved by synthesising a GnRH-KLH conjugate, which was able to induce a systemic anti-GnRH antibody response (Gebril et al., 2014), however it is unclear how long antibody titres were sustained following immunisation.

Bacterial and viral vectors

Bacterial ghosts are a non-living vaccine delivery system which targets both the systemic and mucosal immune systems present in the GI, respiratory and reproductive tracts. These 'ghosts' are essentially the empty envelopes of bacteria following the extrusion of their cell contents (Witte et al., 1992), the resulting empty envelopes share the antigenic and immunostimulatory characteristics of the living bacteria, imparting them with intrinsic adjuvant properties. These bacteria can be made to express the target protein for vaccination, and the resulting antigen will still be present on the bacterial ghost following extrusion of the cell contents. A distinct advantage of this system is that bacterial ghosts are extremely stable during storage and do not require refrigeration (Walcher et al., 2008). In addition, bacterial ghosts may express very large proteins (>600 amino acids) and as such, multiple epitopes of antigens can be simultaneously presented (Eko et al., 2004; Walcher et al., 2004).

As bacterial ghost-delivered vaccines can be administered via a bait or an aerosol (Katinger et al., 1999), they have enormous potential for use in the delivery of an oral contraceptive vaccine for the sterilisation of pest rodents. Initial work using this technology for the immunocontraceptive control of brushtail possums through vaccination against zona pellucida (the coating of the egg) surface peptides is underway (Walcher et al., 2008).

Another potential delivery strategy is to use viral vectors. This involves engineering a virus to deliver the antigen that would otherwise be delivered via injection, resulting in subsequent host immune response against the antigen and therefore fertility reduction. Viral vectors provide the opportunity for highly species-specific delivery, along with a potential means for self-dissemination of the immunocontraceptive strategy where the virus can be spread naturally among individuals following primary infection. Such a strategy would be clearly advantageous in facilitating widespread

rodent control in remote and inaccessible areas. In addition, virus-vectored vaccines may be delivered parenterally using an aerosol to confer a distinct immune response (Bolton et al., 2016), though at this stage no virally vectored immunocontraceptives which can be delivered orally, transcutaneously or nasally have been developed.

Research in Australia resulted in proof of concept for virally vectored immunocontraception, in particular using murine cytomegalovirus (MCMV) and ectromelia virus (ECTV) for delivery of zona pellucida antigen to mice. A proof of concept study was conducted by Jackson et al (1998) and for virally vectored immunocontraception with success first achieved in female mice using a recombinant ECTV expressing mouse zona pellucida subunit 3 glycoprotein (mZP3) from a synthetic poxvirus early–late promoter (Jackson et al., 1998). A single injection of the virus caused infertility in 70% of mice and sub-fertility in the remaining animals. Higher levels of infertility were later attained by others using recombinant MCMV to express the mZP3 antigen (Redwood et al., 2005). In long-term trials, a single intraperitoneal injection of the recombinant MCMC/mZP3 preparation induced infertility in all treated mice within 3 weeks, with sterility persisting for up to250 days (Lloyd et al., 2003). Results also indicated that wild populations of Australian mice should be susceptible to an immunocontraceptive MCMV. There are no published reports of virally vectored immunocontraceptive strategies having been developed specifically for rats.

While lab trials of primary inoculation did cause effective reductions in fertility, further work-up of the technology for field application proved troublesome. The major hurdle has been attenuation of the virus, despite normal replication in culture, and poor transmission of the recombinant virus limited its efficacy as a disseminating product for mice (Hardy et al., 2006). Although a focused research effort was undertaken over 15 years, issues with dissemination, penetrance and attenuation of the virus could not be effectively resolved (Magiafoglou et al., 2003). In addition, there are regulatory, safety and ethical issues associated with replicating vectors, and with the concept of releasing a genetically modified virus into the wild without the option of ‘reversing’ this release. Public concerns revolve around development of resistance, but in particular potential for the viral vector to infect and cause infertility in other species.

Presently such concerns appear to have dampened the feasibility of viral vectored immunocontraceptives and outweighed the potential benefits; despite attaining proof of concept and promising data, the research into further development of virally vectored immunocontraception, for the time being, has been halted. The concept of a virally vectored fertility agent is still a promising one and perhaps its full potential has not yet been conceived of, let alone explored in practice—whether in combination with immunocontraception or other novel strategies discussed here. Genetic engineering is progressing at a rapid pace, and as we learn more about each species’ genome it is possible that novel techniques will be developed to ensure the appropriate ‘checks and balances’ that will allow virally vectored wildlife contraception to be a safe and

ethical technology. On the other hand, a deeper understanding of the species-specific mechanisms of reproduction could reveal novel antigens that are completely unique to a single species, making virally vectored delivery of such an antigen a less controversial endeavour, eliminating the potential for functional cross-immunisation of another species. Thus it is paramount that researchers and funding bodies maintain an open mind toward opportunities that may revive the untapped potential of virally vectored fertility control, but the applicability of viral vectored immunocontraceptives (in its present state) to pest animal fertility control in the immediate future appears minimal. Although they have great potential for species specificity, there is still a small possibility that a virally vectored immunocontraception may affect an off-target species which is also susceptible to both the virus and the vaccine. In the context of Lord Howe Island, strict biosecurity measures such as foot baths and thorough cleaning of all outdoor equipment could be implemented to prevent the inadvertent spread of an immunocontraceptive virus to the mainland.

Species specificity and bait stations

Species specificity of any contraceptive, sterilant or toxicant for the control or eradication of a pest species is of paramount importance. The impacts of an immunocontraceptive on off-target species depend strongly on a) the type of antigen used and b) the delivery and dissemination method used to target the desired species. Using a highly species-specific antigen alleviates to some extent the pressure for it to be delivered in a species-specific manner, although both are desirable. The reverse applies whereby a more generic vaccine can be very safe if delivered very selectively, i.e. by trapping and injection, or by delivery of oral baits using innovative species-targeted stations, or via a species-specific viral vector. In many situations, species specificity of a contraceptive agent or toxin is undesirable as it would seriously restrict the potential application of the product, and as such a far more practical approach to ensuring species specificity is through the use of bait stations which exploit the physical and behavioural characteristics of the target animal at the exclusion of most, if not all of the off target species.

Bait stations serve a variety of purposes and provide several advantages including the protection of baits from environmental degradation, providing an enclosed area for the target species to feel comfortable and safe to feed for extended periods, excluding the majority of non-target species such as human children, pets and native animals, prevention of spillage and release into the environment, and allowing monitoring of how much of the bait is being consumed (Vantassel et al., 2006). Several resetting toxicant delivery systems have been developed to overcome issues associated with sublethal induced bait shyness, off- target dosing, bait degradation and the need to repeatedly replenish baits (Blackie et al., 2014). These devices exploit the grooming behaviours of the target species (in these cases stoats, weasels and possums in New Zealand), delivering a highly palatable toxic paste to the abdomen once the target

animal has activated several species-specific triggers. These stations are able to deliver between 100-500 doses of the toxicant, removing the need to continually re-set or re-load the bait station, and combined with their species specificity could prove extremely useful in delivering contraceptive/sterilant baits formulated for oral consumption.

Several coatings have also been developed to increase the longevity of baits while they are exposed to the elements (Blackie et al., 2014). These coatings preserve the integrity of the bait for up to 5 months, and in most cases also increase palatability. An example of this technology has been registered for use in New Zealand for the control of possums (Pestoff® Waxed Possum Bait). It is likely that similar coating technologies will need to be used for deployment of any oral baits used for fertility intervention and this will need to be taken into account at the formulation stages.

4. Lethal toxins for rodent eradication

While the use of lethal toxins for the control and eradication of pest rodents is an established and proven strategy, innovations around the specificity and efficacy of lethal toxins are being actively explored, in particular with regard to minimizing the risks and impacts on human health, domestic animals and wildlife. These improvements can occur at three distinct levels: identification and development of novel toxic compounds (for example norbormide), the formulation and encapsulation of existing compounds for palatability, stability and specific uptake (for example zinc phosphide and cellulose) and the delivery strategy used to distribute the toxin in a targeted manner. We report here on some new developments pertaining to improved formulation of existing rodenticides (excluding the anti-coagulants), and on novel and emerging toxins that may become available for use in the near future.

New developments in formulation of existing rodenticides

Calciferols (ergocalciferol and cholecalciferol) function by increasing the intestinal absorption of calcium and resorption of bone minerals, leading to a hypercalcaemia, osteomalacia and calcification of the blood vessels and soft tissue, causing death within 3-4 days of ingestion. The effects are not species-specific and there is no antidote available beyond supportive care. Cholecalciferol has been revisited lately and new formulations developed; these have achieved good control of rodents (Eason et al., 2010) however there are still significant concerns about primary off-target poisoning, particularly of birds (Swenson and Bradley, 2013). Careful design of bait stations would be paramount to ensure target species uptake. It has also recently been suggested that the combined use of cholecalciferol with an anticoagulant could have a synergistic rodenticidal effect, and may be useful in areas where

anticoagulant resistance has developed (Blackie et al., 2014; Endepols et al., 2017).

Zinc phosphide is an acute poison that has been used extensively in Australia to control mouse plagues in commercial cropping environments, and in the USA for various species of rodents. The compound releases toxic phosphine gas once ingested, which then enters the bloodstream and results in acute heart failure and organ damage. This mode of action is not specific to rodents and is believed to be more toxic to birds than to rodents, thus posing a high risk to non-target species (Bildfell et al., 2013). Disadvantages of this rodenticide include a short shelf life due to rapid degradation of zinc phosphide, and the development of bait shyness due to its acute toxicity (Jacob et al., 2010). The tendency to generate bait avoidance requires a period of pre-baiting, adding logistical challenges and additional costs to an eradication setting. A microencapsulated formulation of zinc phosphide is in development; the hope is that this would make the compound undetectable by rodents and thus avoid the development of bait shyness, as well as adding shelf/environmental stability (Brown et al., 2007).

A combination of powdered corn cob and cellulose has been marketed for several years as a 'natural' rodenticide. Some formulations also contain molasses as an attractant. The mechanism of action is not entirely clear but it is likely that the components block water absorption in the rodents' digestive tract, resulting in dehydration and ultimately death from circulatory shock within a few days. As the bait works directly on the digestive physiology of the animal there is believed to be no risk of secondary poisoning or bioaccumulation. The effectiveness of these baits has not been examined conclusively, nor have they been successfully used in eradication programs. In one trial, no-choice tests of cellulose based rodenticides appeared to successfully kill mice, however it was not clear whether this was due to bait efficacy or starvation due to low palatability of the baits – bait consumption was extremely low in choice trials against normal food pellets and did not result in deaths, deeming the product unsuitable for rodent control (Schmolz, 2010). Multiple patents pertaining to lethal rodent control have been filed in recent years that appear to be based on the same principle as the powdered corn cob/cellulose baits, varying in composition presumably to maintain environmental stability and be more attractive to rats and mice. The majority of these are authored by researchers or biotechnology firms based in China; studies reporting on the laboratory or field efficacy of these new baits are lacking but may emerge in the near future.

New toxins in development for rodenticide use

Although known to be a Rattus-selective toxin for over 50 years, norbormide is now re-emerging as a potential new strategy for rat control in combination with novel technologies and approaches that may overcome its low palatability (Campbell et al., 2015). Norbormide is a calcium channel blocker; it appears to be 100 times more toxic

to rats than it is to other mammals and to birds (Roszkowski, 1965). In rats, the compound causes a lethal activation of mitochondrial permeability transition mediated by the peripheral benzodiazepine receptor, and presumably, due to a subtle species-specific structural difference of this receptor (Zulian et al., 2011). A combination of inherently low attractiveness and acute lethality results in poor uptake by rats and bait shyness – mortality occurs within minutes of bait consumption, but a sublethal dose deters rats from ever consuming the bait again (Nadian and Lindblom, 2002). Thus, recent research has focused on reviving the potential of norbormide as a highly effective and species-specific rodenticide by synthesising prodrugs that will be consumed by the rat in an inactive form and subsequently converted to active norbormide (Choi et al., 2016; Rennison et al., 2012; Rennison et al., 2013b). This would result in a more delayed but still highly lethal effect, less likely to lead to bait aversion. At the same time improvements to palatability are being sought through various taste-masking strategies. Several new compounds have been evaluated and have shown promising results with respect to delaying onset of symptoms and palatability. Most recently, fatty acid derived pro-toxicants of norbormide and its derivatives have been designed and laboratory experimentation revealed significantly higher palatability and efficacy than norbormide (Choi et al., 2016). One of these compounds has now been selected for use in field trials with the aim of further product development and commercialisation. This is an exciting development and, if the commercialisation phase is successful, its availability will have significant transformative impacts on rodent control and eradication, given the unique species-specificity of this rodenticide and likely low-risk profile with regard to off-target and secondary poisoning. Although extensive field trials are still required to demonstrate efficacy and validate safety for wildlife and human exposure, a norbormide derivative rodenticide is tentatively estimated to become available for use around 2020 (Campbell et al., 2015). It should be emphasised that in the context of rodent eradication on Lord Howe Island, a strategy which is equally lethal for both rats and mice would be preferable, and should norbormide be utilised for the eradication of rats on Lord Howe Island an alternative program would need to be employed for the concurrent eradication of mice.

A new class of active ingredients has been in the spotlight recently as the focus of more humane lethal pest control. Methaemoglobin-inducing compounds are currently available for a wide range of non-native vertebrates including pigs, dogs, foxes and cats. The compounds induce the formation of methaemoglobin, resulting in reduced capacity of blood to carry oxygen followed by respiratory depression and death. Para-aminopropiophenone (PAPP) has been approved for wild dog and fox control in Australia. It is considered a humane toxin with a low risk of bioaccumulation, with the added advantage of having an antidote available to be used in case of off-target poisoning of domestic animals. However, rodents have been reported to have a high methaemoglobin reductase activity after PAPP treatment (Scawin et al., 1984) rendering them much less sensitive to the compound. Novel derivatives are being developed currently to overcome this hurdle, in an attempt to synthesise an effective

PAPP-like rodenticide. Some of these analogues have shown promise and appear to be more toxic in rodents, however these promising in vitro results have thus far not translated into successful in vivo outcomes (Rennison et al., 2013a). Further research is underway to improve toxicity and potency of PAPP analogues for rodents.

Proposed novel toxins

Several sources of compounds that may serve as novel rodenticidal toxins have been proposed via brief references in the scientific literature or patent applications; these are largely at the idea stage or, at most, underpinned by empirical evidence of potential efficacy but not yet proved as viable strategies. To our knowledge, none of the proposed toxins described in this section have entered the formulation or commercialisation phases and it is not clear if their proponents are pursuing further investigation and development of these compounds.

A single patent describes the novel concept of using tetrodotoxin, the lethal toxin contained in pufferfish, as a rodenticide (2015). Tetrodotoxin is a paralysis-inducing neurotoxin but is broken down during fermentation, allowing toxin-containing fish to be consumed as a delicacy in Asian countries (Anraku et al., 2013). The proposition is that byproduct of this consumption, i.e. pufferfish offal, can be exploited to extract a highly palatable toxin for use as a rodenticide. There are no available data on the efficacy or specificity of this formulation; presumably species-specificity would be achieved through formulation of the bait for specific uptake by rodents only, since tetrodotoxins are not species-selective in their neurotoxic activity (Lago et al., 2015) and could pose a significant risk for off-target and secondary poisoning.

Another patent proposes the use of isolated Yersinia murine toxin polypeptide from *Yersinia pestis* or an analogue thereof as a rodenticide (Oyston and Clark, 2010). While the toxin is known to kill rodents, no data are currently available to indicate the safety and support the safe use of this toxin in its purified form. There is expected to be a high risk of rodents developing an immune response and subsequent resistance to the peptide due to antigenicity of the toxin.

Researchers in New Zealand have identified several native plants that are highly toxic to introduced rodents and have made progress on extraction of the toxic compounds, potentially moving towards formulation of these compounds for use as rodenticides following the characterisation of their chemical structures and modes of action (Blackie et al., 2014). The plants tutu (*Coriaria arborea*), karaka (*Corynocarpus laevigatus*) and kowhai (*Sophora microphylla*), have been identified as potential sources of novel toxicants. For example, parts of the tutu plant contain such a high concentration of the tutin toxin that 100 g of plant material contains enough tutin to cull around 3500 mice. This seems to be a very promising direction for novel lethal rodent eradication strategies but is expected to take several years to come to fruition.

5. Conclusion

There exists a vast and comprehensive field of contraceptive research which may be exploited and adapted for use in the control of pest rodent species on isolated islands such as Lord Howe. In situations such as this where there are no native mammalian species to be affected by off-target exposure to contraceptive agents, the concerns and logistical constraints surrounding the need for species specificity are markedly reduced.

The further development of immunocontraceptive strategies is particularly important for several reasons. Immunocontraceptives have the potential to confer life-long infertility following a single exposure to the vaccine, and by targeting species-specific antigens, the risk of off-target species being affected is markedly reduced. Of particular note is the zona pellucida vaccine which can be developed in a species-specific manner (Duckworth et al., 2008) and has been shown to render ungulate species infertile for several years following only one treatment (Gray et al., 2010). In short-lived species such as rats and mice, this may well render the animal infertile for their entire life-span.

The greatest barrier to the use of fertility interventions for the eradication of pest rodents is that there have been very few resources directed towards adapting the currently available immunocontraceptives and gonadotoxins for delivery via oral, transcutaneous or mucosal routes, and as such the only commercially available formulation appears to be the gonadotoxin ContraPest®, a product which has not yet been approved for use within Australia. It should also be noted that this product has been developed for use in urban environments; while it is claimed not to affect much larger mammals such as domestic pets and humans (presumably by virtue of targeted baiting stations and the small doses delivered), it is not known whether it could affect the fertility of smaller non-mammalian animals that are present on Lord Howe Island. Another family of gonadotoxic contraceptive agents well worth revisiting in the context of rodent fertility control are ornidazole and other glycolysis-inhibiting compounds. These drugs induce complete, albeit temporary infertility following oral administration (Cooper and Yeung, 1999; Oberlander et al., 1994). Innovations pertaining to improved formulations for delivery, dissemination and prolonged duration of efficacy will be crucial for progress in this field. Particularly in rodents, where delivery by injection is not an option, even the most effective contraceptives and sterilants cannot be deployed unless an effective means of delivery is developed alongside.

Although likely to be controversial and presently in early stages of development, genetic engineering for gender distortion and sterility could provide a transformative and highly effective approach to limiting the reproductive capacity of rodent populations and ongoing dissemination of infertility, without the risk of environmental contamination or off-target poisoning by rodenticides. These strategies are

particularly appealing for island scenarios where modified populations are geographically isolated and have a realistic chance of eradication, with minimal risk for dissemination of the technology beyond its intended target population. It is expected that such strategies will be highly species-specific and pose minimal risk to off-target species; clearly, stringent safety and ethics frameworks would need to be established prior to release. While genetic approaches will likely be highly cost-effective for eradication projects in the long term, significant investments into planning, modelling and biosecurity will be essential, as well as the navigation of regulatory requirements and community engagement to gain acceptance of genetic engineering strategies as a legitimate and safe tool for ecosystem management.

Development of new toxicological approaches to rodent eradication is underway, however many of these are in fact older compounds that have experienced a revived interest in recent years, and investments have been made in upgrading the safety and attractiveness of these compounds to make them relevant to current pest control standards. Since the majority of these are not species-specific, many still present concerns about off-target and environmental impacts. Successful development of effective *Rattus*- and *Mus*-specific toxins would have major implications for conservation and in particular eradication programs, and we expect that these will continue to be the focus of research for lethal pest control. With ever increasing knowledge of the genomes and proteomes of individual species, there are numerous opportunities to take advantage of the improved understanding of species-specific differences in physiology that might reveal new candidate targets for pest control and eradication.

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